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Yeast Proteins Associated With Microtubules In Vitro and In Vivo

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Conditions were established for the self-assembly of milligram amounts of purified *Saccharomyces cerevisiae* tubulin. Microtubules assembled with pure yeast tubulin were not stabilized by taxol; hybrid microtubules containing substoichiometric amounts of bovine tubulin were stabilized. Yeast microtubule-associated proteins (MAPs) were identified on affinity matrices made from hybrid and all-bovine microtubules. About 25 yeast MAPs were isolated. The amino-terminal sequences of several of these were determined: three were known metabolic enzymes, two were GTP-binding proteins (including the product of the *SAR1* gene), and three were novel proteins not found in sequence databases. Affinity-purified antisera were generated against synthetic peptides corresponding to two of the apparently novel proteins (38 and 50 kDa). Immunofluorescence microscopy showed that both these proteins colocalize with intra- and extranuclear microtubules in vivo.

INTRODUCTION

During the life cycle of the yeast *Saccharomyces cerevisiae*, the microtubule cytoskeleton undergoes highly regulated changes in organization and function (Byers, 1981; Adams and Pringle, 1984; Kilmartin and Adams, 1984; reviewed in Barnes *et al.*, 1990). During mitotic growth, the microtubules interconvert between aster-like cytoplasmic arrays (of undetermined function) in unbudded cells and elongating mitotic spindles responsible for separating and segregating the chromosomes during division. Also during mitosis, an extranuclear microtubule cytoplasmic array is responsible for nuclear movement and division (in yeast, the nuclear membrane does not break down during mitosis) (Huffaker *et al.*, 1988; Jacobs *et al.*, 1988). This interconversion between aster-like array and elongating mitotic spindle is coordinated with the growth of the developing progeny cell or bud.

The microtubule cytoskeleton not only cycles through these two types of tubulin-containing structures but can be triggered to form still different structures with distinct functions during mating. In zygotes, microtubules are responsible for bringing nuclei together during karyogamy (Huffaker *et al.*, 1988; Jacobs *et al.*, 1988). Before the fusion of cells of opposite mating type, the microtubule cytoskeleton in each of the mating cells reorients so that the cytoplasmic microtubules are projected in the direction of the mating partner. Once the cell walls and plasma membranes have fused, the cytoplasmic microtubules associated with each nucleus function in a coordinated fashion to achieve nuclear fusion of the newly formed zygote. As the diploid cell reinitiates mitotic growth, the organization and function of the microtubules are again reorganized to follow the mitotic program.

A third microtubule cytoskeletal role is in meiosis where the meiotic spindle participates in both the reductional and equational meiotic divisions necessary to generate haploid progeny (Byers, 1981). After the divisions of meiosis, the cytoskeleton is arrested and prevented from undergoing mitotic assembly cycles during the suspended cell growth of the haploid spore phase of the life cycle.

In addition to the α- and β-tubulins (the major protein constituents), the microtubule cytoskeleton consists of other proteins collectively referred to as microtubule-associated proteins (MAPs). MAPs were originally identified in mammalian brain tubulin cycling experiments as proteins that would co-pellet with assembled microtubules (Olmsted, 1986; Vallee and Collins, 1986). Despite the fact that MAPs were first identified a number of years ago and have been the subject of extensive biochemical analyses, very little direct evidence for the specific roles of MAPs in vivo is available.

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In *S. cerevisiae*, there is only a single β-tubulin gene, *TUB2* (Neff *et al.*, 1983) and just two α-tubulin genes, *TUB1* and *TUB3* (Schatz *et al.*, 1986a,b); therefore, it is reasonable to assume that the complex and coordinated changes of the microtubule cytoskeleton during mitosis, meiosis, or mating are not due solely to this limited heterogeneity of the tubulin proteins but more likely to the activities of various MAPs. MAPs may play a structural role and participate directly in an assembly process, they might regulate a critical step in an assembly process, they might mediate interactions between microtubules and other cell constituents, or they might play some as yet unidentified roles. Therefore, to understand how the microtubule cytoskeleton functions, not only is it important to understand the tubulin proteins themselves, but also to identify the MAPs and to study these proteins individually and collectively both in vivo and in vitro in association with microtubules.

As a result of several different genetic selections and screens, many mutations have been isolated in the three yeast tubulin genes (Thomas *et al.*, 1985; Huffaker *et al.*, 1988; Schatz *et al.*, 1988; Stearns and Botstein, 1988). In addition, accessory proteins important for microtubule function have been genetically identified. Examples include the proteins encoded by the *CIN1, 2,* and *4* genes (Hoyt *et al.*, 1990; Stearns *et al.*, 1990a); the *BIK1* gene (Berlin *et al.*, 1990); and the *KAR1, 2,* and *3* genes (Conde and Fink, 1976; Rose and Fink, 1987; Rose *et al.*, 1989; Meluh and Rose, 1990). Collectively, these studies have helped to define the roles of microtubules in yeast and have expanded the repertoire of proteins known to be important for microtubule function. Although much progress has been made on the genetics of the yeast microtubule cytoskeleton, the utility of yeast for studies on tubulin and associated proteins has been limited by the relatively small amount of biochemical work that has been done thus far with yeast microtubules.

A method using taxol-stabilized microtubules as an affinity matrix to identify MAPs from *Drosophila* embryos was developed by Kellogg *et al.* (1989). In that work, the authors demonstrate that a complex profile of proteins binds to the microtubule affinity column and that most likely a majority of the proteins interact in some manner with the cytoskeleton in vivo. They found that 20 out of 24 polyclonal antisera generated to 24 different binding proteins stain microtubule structures in immunofluorescence experiments. In the work presented in our paper, we report a procedure that reproducibly yields milligram quantities of in vitro assembly-competent *S. cerevisiae* tubulin and the use of affinity chromatography using in vitro polymerized microtubules to identify and purify MAPs from the yeast *S. cerevisiae*.

MATERIALS AND METHODS

Materials

DEAE-cellulose (DE-52) was obtained from Whatman Ltd. (Clifton, NJ). Ammonium sulfate was purchased from Aldrich Chemical Co (Milwaukee, WI). Sephadex G-25 was obtained from Pharmacia, Inc. (Piscataway, NJ). All other chemicals were analytical reagent grade from Sigma (St. Louis, MO) unless specified. Five hundred-micrometer glass beads were purchased from Sigma and Biospec Products, Inc. (Bartelsville, OK). 10-deacetylbaccatine III was a gift from Dr. Daniel Guenard (ICSN-CNRS, Gif-sur-Yvette, France). Taxol was a gift from Dr. Matthew Suffness (National Institutes of Health, Bethesda, MD).

Purification of S. cerevisiae Tubulin and In Vitro Characterization

Measurement of Protein Concentration. Protein concentration was measured by the method of Lowry *et al.* (1951) on samples that had been precipitated with trichloroacetic acid (Peterson, 1977). Bovine serum albumin (BSA) and ovalbumin (Sigma) were used as protein standards.

Gel Electrophoresis and Protein Sequencing. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was carried out with 8.5% acrylamide gels. Proteins were visualized by Coomassie Blue staining or by silver staining (Accurate Chemical and Scientific Corp., Westbury, NY).

Two-dimensional electrophoresis was performed according to O'Farrell (1975). For the first dimension, 1.6% pH 4–6 and 0.4% pH 3–10 ampholines (BioRad, Richmond, CA) were used. The second dimension was a standard SDS 12.5% polyacrylamide gel (Laemmli, 1970). The proteins resolved in this way were then electroblotted onto ProBlott membrane (Applied Biosystems, Foster City, CA) in 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid), pH 11.0/10% methanol for 30 min at 170 mA. The blots were stained for 1 min in 1% Coomassie Blue R250 in 40% methanol/1% acetic acid and destained in 50% methanol/10% acetic acid for 5 min. After destaining, the blots were rinsed extensively with water, air dried, and stored at −20°C. The protein spots of interest were excised and subjected to amino terminal sequencing in a 470A gas phase sequencer (Applied Biosystems) and the amino acids were analyzed in a 120A on-line PTH analyzer (Applied Biosystems).

Immunoblotting. For immunoblot experiments, proteins were transferred from SDS polyacrylamide gels to nitrocellulose filters (Schleicher and Schuell, Keene, NH) as described by Burnette (1981). Depending on the experiment, any of the following antibodies were used. To detect the two yeast α-tubulins, *TUB1*p and *TUB3*p, antiserum 345 (a gift from Peter Schatz, MIT, Cambridge, MA) was used at a dilution of 1:1000. To detect yeast β-tubulin, either of two antisera was used: YOL 1/34 (Kilmartin *et al.*, 1982) (Serotech, Oxford, UK) at a dilution of 1:300 and a yeast β-tubulin-specific antibody (a gift from Frank Solomon, MIT, Cambridge, MA) (Bond *et al.*, 1986) used at a dilution of 1:3000. The primary antibodies were detected using the Amersham Chemiluminescence (ECL) detection system.

Immunofluorescence Coverslip Assay. Immunofluorescence experiments to visualize the microtubule assembly product on polylysine-coated coverslips were carried out according to Mitchison and Kirschner (1984a). Briefly, microtubules were assembled as described below and the assembly reaction fixed in PMI buffer (100 mM piperazine-*N,N'*-bis[2-ethanesulfonic acid] [PIPES], pH 6.8, 10 mM $MgSO_4$, 2 mM ethylene glycol-bis[β-aminoethyl ether]-*N,N,N',N'*-tetraacetic acid [EGTA], 1 mM GTP, 5 mM dithiothreitol [DTT]) containing 0.5% (vol/vol) glutaraldehyde (Polysciences Inc., Warrington, PA) for 5 min. After fixation, the assembly reaction was diluted ($1/2 \times 10^4$) into PMI buffer without glutaraldehyde and centrifuged at 25 000 × *g* in an HB4 rotor (Sorval, Dupont Instruments) using a modified Corex 8441 tube containing a polylysine-coated coverslip. For immunostaining, different tubulin-specific antisera were used: the yeast tubulin-specific antibody (Bond *et al.*, 1986) at a dilution of 1:500, YOL 1/34 (Kilmartin *et al.*, 1982) (Serotech) at a dilution of 1:50, or Amersham monoclonal antibody N.357 at a dilution of 1:250. Fluorochrome-conjugated secondary antibodies (either fluorescein isothiocyanate or rhodamine) and nonconjugated secondary antibodies were purchased from Cappel/Organon Teknika (West Chester, PA).

Electron Microscopy. Samples for electron microscopy shown in Figure 1B were prepared by fixing the assembly reaction (100 μl) with 2

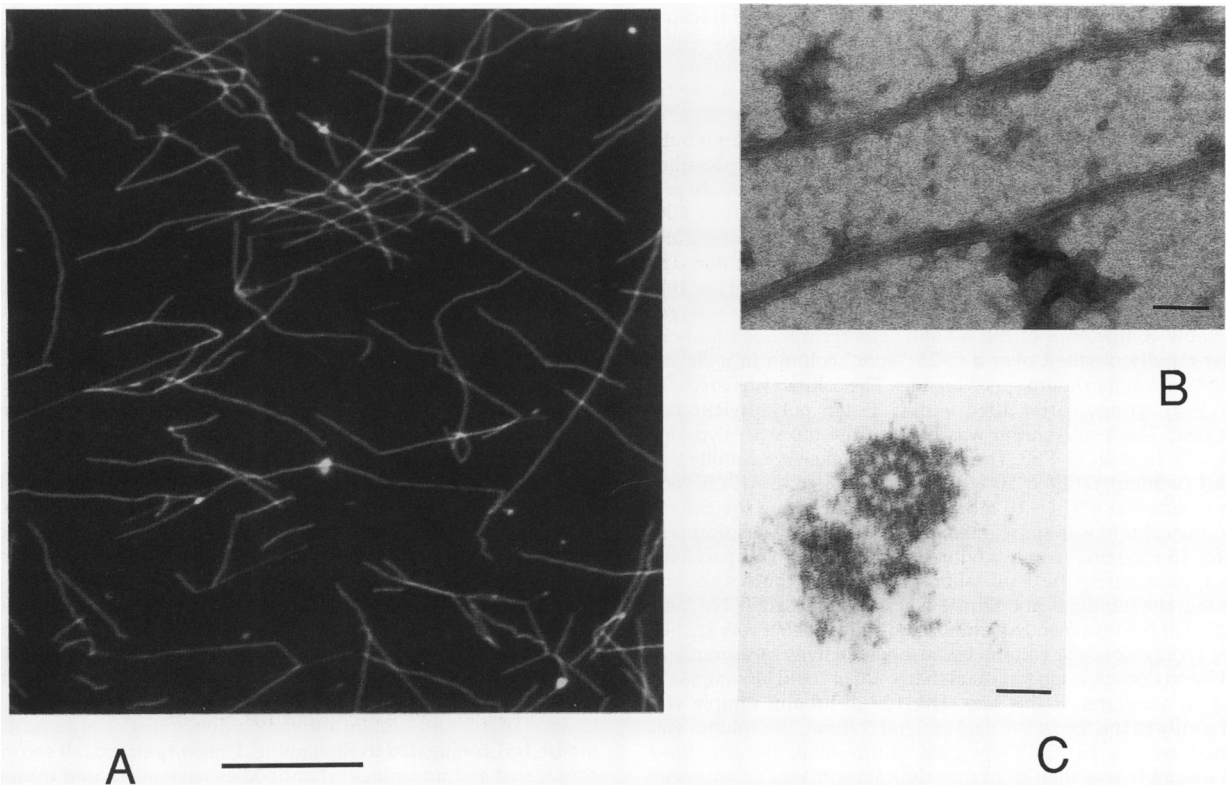

Figure 1. In vitro assembled yeast microtubules. (A) Immunofluorescence of assembled yeast microtubules. Glutaraldehyde-fixed microtubules were sedimented at 25K × *g* onto coverslips and the microtubules were visualized by immunofluorescence with an anti-tubulin antibody to fluorochrome-conjugated secondary antibody. The bar is a 10-μm size marker. Electron microscopic analysis B and C. (B) Negatively stained yeast microtubules at 111 750× magnification. The bar is ~74 nm. (C) Cross-section of yeast microtubule in thin section at 453 000× magnification. The bar is ~22 nm.

ml of PMI buffer containing 1% (vol/vol) glutaraldehyde. The fixed samples were placed on Formvar-coated carbon stabilized grids (Polysciences, Warrington, PA), negatively stained with 1% uranyl acetate, and examined using a Zeiss 109 microscope at 80 kV (Zeiss, Thornwood, NY).

The samples shown in Figure 1C were prepared by pelleting the microtubule assembly reaction after glutaraldehyde fixation onto polylysine-coated plastic coverslips (Thermanox, #5413 Nunc, Inc., Naperville, IL) in the same way described above for the immunofluorescence coverslip assay. To prevent the microtubules from floating off of the coverslip during the rest of the sample preparation, the coverslip and attached microtubules were covered by a layer of 1% low melt agarose (FMC, Rockland, ME) in PMI buffer. The coverslip/microtubules/agarose sample was then processed for electron microscopy by the method of Evans *et al.* (1985), except that PMI buffer was used throughout the procedure and the embedment medium used was Spurr's resin (Polysciences, Inc., Warrington, PA). The sections were examined using a Philips EM300 microscope at 80 kV.

Bovine Brain Tubulin. Phosphocellulose purified bovine brain tubulin was prepared essentially as described by Mitchison and Kirschner (Mitchison and Kirschner, 1984b).

Yeast Tubulin Purification Protocol. *Crude Extract.* Five hundred grams of cells (wet weight) were washed once in ice-cold distilled water. The cells were resuspended in an equal volume of PMI buffer containing GTP, DTT, aqueous protease inhibitors, and *Nα-p*-tosyl-L-arginine methyl ester (TAME). Cell lysates were prepared in PMI buffer. Immediately before lysis, a 1:500 dilution of the aqueous protease inhibitor cocktail (0.5 mg/ml of each of the following: antipain, leupeptin, pepstatin A, chymostatin, and aprotinin) was added to the PMI buffer. Also added just before lysis was TAME to a final concentration of 1 mM. Immediately after lysis, a 1:100 dilution of the following solution was added: 0.2 M (phenylmethylsulfonyl fluoride), 1 mM benzamidine HCl, and 0.5 mM phenanthroline, dissolved in isopropanol. TAME was only added to the lysis buffer and was not used in column buffers. For all column buffers used throughout the purification, the aqueous protease inhibitor cocktail and the isopropanol protease inhibitor cocktail were each used at a 1:500 dilution of the stock solutions.

The cells were lysed by disruption in a stainless steel glass bead homogenizer (Bead-Beater, Biospec, Bartelsville, OK) using 500-μm acid-washed glass beads and maintaining the temperature at 2–4°C with an ice-water jacket around the bead beater chamber. The cells were homogenized for a total of 12 min in 12 cycles of 1 min homogenization followed by a 2-min cooling interval. The extent of cell lysis was evaluated by examining the homogenate under a phase microscope and was judged to be >95%. After cell lysis, the isopropanol protease inhibitor cocktail was added and the crude extract was centrifuged at 100 000 × *g* for 60 min using a 45Ti rotor (Beckman, Fullerton, CA).

DEAE Chromatography. The high speed supernatant was brought to 0.18 M NaCl and 10% glycerol by the addition of solid NaCl and 100% glycerol. Fifty milliliters of DE-52 (Whatman, Clifton, NJ) resin (previously equilibrated with PMI and 0.18 M NaCl and 10% glycerol) were mixed into the high speed supernatant in a beaker on ice using a platform rotator. After 30 min this mixture was poured into a 2.5 × 13-cm column, the column was washed with 5 volumes of buffer (PMI + 0.18 M NaCl + 10% glycerol), and the tubulin was eluted with a single bump of PMI buffer containing 10% glycerol and 0.6 M NaCl. Eight-milliliter fractions were collected.

Ammonium Sulfate Fractionation. To each DE-52 fraction that con-

tained tubulin (determined by SDS gel electrophoresis, 4–5 fractions of 8-ml each), crystalline ammonium sulfate was added to ~60% saturation (375 mg/ml) with constant gentle mixing. The tubulin precipitated by ammonium sulfate remains competent for assembly for ~1 mo if it is maintained as a precipitate at 4°C.

Desalting the Ammonium Sulfate Precipitate. Because the yeast tubulin is stable in ammonium sulfate at 4°C for ≥4 wk, the following desalting procedure was often used on only part of an ammonium sulfate precipitated fraction. Four milliliters of precipitate suspended in 60% ammonium sulfate solution was transferred to a 15-ml corex tube and centrifuged at 9600 × *g* for 10 min at 4°C in an SS-34 rotor. The pellet was resuspended in 400 μl PMI buffer (without DTT) + 10% glycerol at 4°C. Note, from this point, speed is essential to preserve the assembly competence of the tubulin. The resuspended material was next rapidly desalted over a G-25 "spin" column in a clinical centrifuge spun at maximum speed for 15 s. The column was prepared using a 1-ml syringe barrel fitted with a 35-μm polyethylene bed support disc. The 1-ml column was filled to the top with hydrated Sephadex G-25 resin at 4°C. The resin was extensively equilibrated with PMI (without DTT) + 10% glycerol by washing with several column volumes at 4°C (~10 ml). Just before desalting the protein fraction, the column was spun at maximum speed in the clinical centrifuge for 15 s to remove excess liquid from the resin. This packs the resin to approximately the 1-ml calibration mark on the syringe and ensures that the tubulin is not diluted as it is being desalted. No more than 100 μl of the resuspended ammonium sulfate pellet was applied to a 1-ml syringe desalting column because conductivity measurements of the column flow-through indicated that ≤120 μl could be completely desalted on a column of this size. The 100-μl tubulin sample was applied gently to the top of the packed 1-ml column, the column was suspended inside a 13 × 100-cm glass culture tube, and the tube/column assembly was then spun in a clinical centrifuge at top speed for 15 s at 4°C. The desalted tubulin (~100 μl) was collected in the 13 × 100-cm culture tube. To remove particulates, the desalted tubulin was transferred immediately to 7 × 20-mm tubes and centrifuged at 140 000 × *g* for 2 min at 4°C in a TL100 centrifuge using the TLA100 rotor (Beckman). After this cold centrifugation, the supernatant containing the tubulin was either immediately used in assembly assays or was immediately stored as assembly-competent monomeric tubulin at −70°C.

Storage of Monomeric Tubulin. At this stage, the desalted tubulin can be stored for ≥1 y at −70°C in monomer form without losing assembly-competence. Fifty- or 100-μl aliquots of the supernatants (above) were rapidly frozen by immersion in liquid nitrogen.

Yeast Microtubule Assembly. Using freshly thawed or freshly desalted material, the microtubule assembly reaction was carried out in a TLA100 tube (Beckman) 7 × 20 mm. Fresh GTP was added (to 1 mM) to the tubulin solution at 4°C from a 100 mM stock prepared in PMI (without DTT) + 10% glycerol. Dimethyl sulfoxide (DMSO) (Me_2SO) was added to a final concentration of 10% (vol/vol) from a 100% stock. The reaction was moved from 4°C to a water bath at 27°C and incubated for 20 min. To separate assembled microtubules from unassembled tubulin, the reaction was centrifuged in a TLA100 rotor at 140 000 × *g* for 2 min. For immunofluorescence experiments, the microtubules were processed as described above.

Hybrid Microtubule Assembly. Yeast microtubules were assembled as above and after 20 min; bovine tubulin (purified above) was added to the assembly reaction. Five minutes after the bovine tubulin was added, a step addition of taxol was started. The step addition of taxol was used to avoid the formation of tubulin aggregates. The assembly reaction was first made to 0.15 μM taxol, incubated for 5 min, then raised to 3 μM taxol and incubated for 5 min, then raised to 15 μM taxol and incubated for 5 min, and finally raised to 30 μM taxol and incubated for 5 min. The assembly reaction was then either fixed for immunofluorescence using PMI buffer plus 0.5% glutaraldehyde as described above (at a dilution of $1:2 \times 10^4$) or diluted (1:10) into PMI buffer without glutaraldehyde for stability assays. Then after the incubation in the absence of taxol, the microtubules were fixed in PMI buffer plus 0.5% glutaraldehyde (at a dilution of $1:10^3$ giving a final $1:2 \times 10^4$ final dilution of the assembled material) and examined by immunofluorescence.

Blocking Strategy to Detect Different Regions of Hybrid Microtubules. To investigate the distribution of bovine brain tubulin in the hybrid microtubules, a double-label immunofluorescence experiment was carried out using an antibody "sandwiching" method. This method has been shown to be an effective blocking technique in studies designed to differentiate between distinct tubulin populations in vivo (Schulze and Kirschner, 1987). In our experiments, the analysis involves incubating the hybrid microtubules sedimented as described onto polylysine-coated coverslips first with a yeast tubulin-specific antibody raised in rabbit (Bond *et al.*, 1986) and then incubating with 2° goat anti-rabbit rhodamine conjugated antisera, then incubating with 3° rabbit anti-goat antisera, then incubating with 4° goat anti-rabbit antisera, and then incubating with 5° rabbit anti-goat antisera. The multiple cycles of secondary antibody incubations make a physical barrier against the next tubulin antibody (which recognizes both bovine and yeast tubulins) so that only areas of the microtubule-containing bovine tubulin are now accessible to further antibody binding. The next incubation is the 6° step with Amersham mouse monoclonal anti-β tubulin antibodies (which now effectively only recognize bovine tubulin) and finally 7° goat anti-mouse fluorochrome conjugated antisera were then added. Note that the 2° goat anti-rabbit rhodamine conjugated serum was purified over mouse IgG on sephadex resin to eliminate cross-reactivity to mouse sera, and the goat anti-mouse fluorochrome-conjugated serum was purified over rabbit IgG on sephadex resin to eliminate cross-reactivity to rabbit sera. In this experiment, the regions of the hybrid microtubule containing only bovine brain tubulin are visualized using the second anti-tubulin antibody. The location of this anti-tubulin antibody is determined using a goat anti-mouse IgG conjugated to rhodamine. Unless specified, all secondary conjugated and unconjugated antibodies were purchased from Cappel/Organon Teknika.

Affinity Chromatographic Isolation of S. cerevisiae MAP

Yeast Protein Extract Preparation. Fresh Red Star baker's yeast was purchased in 500-g (wet weight) bricks from Universal Foods Corp. (Oakland, CA). Cell lysates were prepared using ~400 g of cells (wet weight). The cells were washed once in cold deionized water and pelleted using a GSA rotor (Dupont Instruments) at 14 000 × *g*. The washed cells were resuspended in lysis buffer: 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.6, 1 mM $MgCl_2$, 1 mM EGTA, 0.05% TWEEN 20, 0.5 mM DTT. The aqueous and isopropanol protease inhibitor cocktails as well as the TAME were used as described in Crude Extract. Again, the isopropanol protease inhibitor cocktail and TAME were only added to the lysis buffer and were not used in any of the column buffers.

The cells were lysed as described in MATERIALS AND METHODS. The crude extract (~750-ml volume) was centrifuged at 14 000 × *g* rpm for 20 min using a GSA rotor at 4°C. The low-speed supernatant (500 ml) was then spun in a 45 Ti rotor (Beckman) at 100 000 × *g* for 90 min at 4°C. To the high-speed supernatant (260 ml), apyrase (Sigma) was added to a final concentration of 4 units/ml. This was intended to lower ATP levels before column loading. The high-speed supernatant was then loaded at 4°C onto a 150-ml BSA (Sigma) affinity column composed of 75 ml Sepharose CL-6B mixed with 75 ml Affigel 10 (Biorad). This large BSA column was equilibrated with the HEPES buffer containing DTT and the aqueous protease inhibitor cocktail. Running the extract through the 150-ml BSA column greatly reduced background nonspecific binding on the subsequent columns. (The preparation of all affinity columns is described below). The flow-through from the 150-ml BSA column was split in half and one half was then run over a second BSA column (16 ml total volume of 8 ml Sepharose CL-6B mixed with 8 ml Affigel 10) and the other half was run in parallel over a microtubule affinity column (also 16 ml total volume of 8 ml Sepharose CL-6B mixed with 8 ml of Affigel 10). To the half of the high-speed supernatant that was passed over the mi-

crotubule column, taxol was added to a final concentration of 2 μM. The two halves of the high-speed supernatant were recirculated over their respective columns at a rate of 8 ml/h for 16 h.

After binding the yeast protein extract to the microtubule and BSA affinity columns, each column was washed with 150 ml of HEPES buffer containing DTT and the aqueous protease inhibitor cocktail at a flow-rate of 10 ml/h. To the 150 ml of wash buffer that was applied to the microtubule column, taxol was added to a final concentration of 2 μM. Note, 2 μM taxol was included in all buffers run over the microtubule column. After washing the columns, both columns were subjected to three different buffer conditions: HEPES buffer, DTT, and aqueous protease inhibitor cocktail containing nucleotide (5 mM ATP and 2 mM GTP) then buffer containing 0.1 M KCl, and finally buffer containing 0.5 M KCl. In each of the three elution buffers that was applied to the microtubule column, taxol was added to a final concentration of 2 μM. Both columns were eluted with 80 ml of buffer for each elution condition. The flow rate for the elutions was 3 ml/h and 2-ml fractions were collected. The fractions were frozen in liquid nitrogen and stored at −70°C.

To remove the KCl and for subsequent short-term storage, the 16-ml BSA and microtubule affinity columns were then each washed with 100 ml of PMI buffer and stored at 4°C in the presence of 2 mM azide. The large 150-ml BSA column was washed with 200 ml coupling buffer plus 0.5 M KCl and then rinsed with 200 ml PMI buffer plus 2 mM azide and stored at 4°C.

Preparation of Microtubule Affinity Columns. Twenty-seven milligrams of phosphocellulose purified bovine brain tubulin (Mitchison and Kirschner, 1984a) were assembled into microtubules in the presence of taxol, which was added in a stepwise manner to avoid the formation of tubulin aggregates (Schiff *et al.*, 1979; Kellogg *et al.*, 1989). To the 27 mg of tubulin in an 8-ml volume, GTP was added to a final concentration of 1 mM and DMSO was added to a final concentration of 10% (vol/vol). The assembly reaction was carried out at 37°C in four steps of 10 min each. First taxol was added to the reaction to a final concentration of 0.12 μM. Then the taxol concentration was raised to 1.0 μM, 10 μM, and finally to 20 μM for the final reaction step. The pH of the taxol stabilized microtubules was adjusted from pH 6.8 to 7.5 with 2 M KOH to increase the efficiency of coupling to the Affigel 10 resin (Kellogg *et al.*, 1989).

The taxol-stabilized microtubules were then immobilized on 8 ml of Affigel 10 resin mixed with 8 ml of Sepharose CL-6B resin precisely as described in the protocol of Kellogg *et al.* (1989). Sepharose CL-6B was used to enhance the flow properties of the column. The final column bed volume was ~16 ml. The columns were prepared in a BioRad 2.5 × 13-cm column. To construct BSA control columns, 90 ml of 10 mg/ml BSA (Sigma) in coupling buffer was mixed with 90 ml of Affigel 10 resin and 90 ml of Sepharose CL6B (Kellogg *et al.*, 1989) to generate 180 of column resin. Part of this BSA resin was used for the large BSA column used to adsorb nonspecific proteins from extracts and the other part was used for the BSA column run in parallel with the microtubule column.

Buffers. Coupling buffer was comprised of 100 mM PIPES, pH 7.5, 1 mM $MgSO_4$, 1 mM EGTA. HEPES buffer was comprised of 50 mM HEPES, pH 7.6, 1 mM $MgSO_4$, 1 mM EGTA, DTT 0.5 mM, aqueous protease inhibitor cocktail 1:500 dilution of stock.

Synthetic Peptide Synthesis. Peptides were synthesized using conventional solid-phase chromatography methods at Genentech, Inc. (South San Francisco, CA).

Preparation of Amino-Terminal Peptide Antibodies. The following synthetic peptides of the 50- and 38-kDa proteins, respectively, TLSDAHHKLITSHLVNTC and MKCTIPEQQKVILIDEIGC, were coupled to soybean trypsin inhibitor as a carrier protein for the production of polyclonal antiserum in rabbits. The titer of the antiserum produced was monitored by standard (ELISA) techniques (Walter and Doolittle, 1983).

Peptide Affinity Columns and Purification of Anti-Peptide Antisera. To purify the polyclonal anti-peptide antisera, peptide affinity columns were constructed using a matrix of vinyl sulfone agarose (Sigma). Five milligrams of peptide were used for immobilization on the matrix. The lyophilized peptide (5 mg) was resuspended in 2.5 ml of a solution of 0.2 M *N*-methyl morpholine and 1 mM EDTA. This solution was adjusted to pH 8.0 with acetic acid before mixing with the peptide. Vinyl sulfone agarose resin (0.2 g) was used to prepare a 1-ml affinity column. The powdered resin was swollen and washed in water at room temperature. The swollen resin was washed once in 1.0 M potassium phosphate buffer, pH 6.5, twice in 0.5 M sodium carbonate buffer, pH 10.0, then washed again with water, and finally, the resin was washed with the *N*-methyl morpholine/EDTA buffer. The resin was next scraped into a polypropylene 15-ml conical tube and mixed with the resuspended peptide. The atmosphere was replaced with nitrogen for the binding period. The peptide was allowed to bind to the resin for 18 h at room temperature on a gently (to avoid the generation of fines) rocking platform. The unreacted vinyl sulfone groups were then blocked by addition of 5 μl of 14M β-mercapto-ethanol and mixed for 3 more h. The resin was stored in phosphate-buffered saline (PBS) (pH 6.0) with 30 mM azide at 4°C.

Affinity-purified antisera were prepared following the protocol of Drubin *et al.* (1988) as outlined in Pringle *et al.* (1989). The affinity-purified antibodies in PBS/azide/35% glycerol were stored at −20°C.

Immunofluorescence of Whole Yeast Cells. Wild-type yeast (strain DBY 5288) were grown to early log phase (usually 2×10^6 cells/ml). Fixation and immunofluorescence procedures were adapted from Kilmartin and Adams (1984). Best results were obtained when cells were grown in rich medium and fixed by adding 0.78 ml of 37% formaldehyde directly into a 5-ml culture. After 60–90 min, the cells were washed twice with 0.1 M potassium phosphate, pH 7.5, and resuspended in 0.5 ml of this buffer. To remove cell walls, 1 μl of β-mercapto-ethanol and 25 μl of 1 mg/ml zymolyase 100 000 were added. After 40 min at 37°C, the spheroplasts were pelleted using a clinical table top centrifuge and then resuspended in fresh 0.1 M potassium phosphate buffer, pH 7.5. Fifteen microliters of the spheroplast suspension was transferred to each polylysine-coated well on a multiwell slide (Flow Laboratories, McLean, VA). The slides were incubated at room temperature for 20 min and then remaining liquid was aspirated off and the wells completely air-dried for 15 min. The spheroplasts were rehydrated in PBS for 15 min at room temperature. The wells were then blocked with PBS containing 1 mg/ml BSA. All anti-sera were diluted in PBS-BSA. Antibody incubations were 60 min, followed by several washes with PBS. Slides were mounted in 90% glycerol containing *p*-phenylenediamine as described by Kilmartin and Adams (1984). Note, it was essential to follow this protocol and not postfix with methanol/acetone at −20°C to visualize the immunofluorescence staining of the anti-peptide antibodies for the 38- and 50-kDa proteins. The peptide antibodies were used at a dilution of 1:50 in PBS/BSA for these immuno-localization experiments. To visualize the microtubule staining pattern in the double-label immunofluorescence experiments, the anti-tubulin rat monoclonal antibody YOL 1/34 (Kilmartin *et al.*, 1982) (Serotech) was used at a dilution of 1:200 in PBS/BSA. Fluorochrome-conjugated secondary antibodies (either fluorescein isothiocyanate or rhodamine) were purchased from Cappel/Organon Teknika.

Concentrating Proteins. To sequence the small GTP binding proteins bound to the microtubule column, it was necessary to concentrate the column fractions. The peak fractions containing the 25 and 27 kDa GTP binding proteins were pooled (~7 ml total volume) and then protein was precipitated with a chloroform/methanol procedure using ultrapure reagents to avoid *N*-terminally blocking the proteins. The pooled material was divided into 150-μl volumes into separate eppendorf tubes. To each tube 600 μl of methanol was added, the solution mixed with a vortex, 150 μl chloroform was added, the solution mixed with a vortex, 450 μl 18 Ω water was then added, and the solution mixed with a vortex. This mixture was then spun in a microfuge for 2 min. The upper layer was decanted and discarded. The interface contained the protein and was not disturbed. Four hundred fifty microliters of methanol were added to the interface and lower-phase layer remaining in the tube. This single-phase solution was spun for 1 min in a microfuge. The supernatant was decanted carefully while

not disturbing the pellet. The protein pellet was then air-dried and then resuspended in 15 μl conventional protein sample buffer for the sequencing step.

Immunoblotting. See under Purification of S. Cerevisiae Tubulin and In Vitro Characterization for a description. The following antibodies were used: anti-Ypt1p antibody at a dilution of 1:500 (a gift from Nava Segev, Segev *et al.*, 1988), anti-Arf1p antibody at a dilution of 1:500 (a gift from Richard Kahn, Stearns *et al.*, 1990b), and anti-RAS antibody monoclonal Y13-259 at a dilution of 1:800 (a gift from Art Levinson, Genentech, Inc.). The affinity-purified anti-peptide antibodies generated against the 38- and 50-kDa microtubule-binding proteins were used for immunoblot experiments at dilutions of 1:1000 and 1:25 000, respectively. To detect tubulin leaching off of the microtubule column, YOL 1/34 (Kilmartin *et al.*, 1982) (Serotech) was used at a dilution of 1:300.

RESULTS

The overall design of these experiments can be summarized as follows. We isolated yeast tubulin in assembly-competent form. The yeast microtubules could be stabilized with taxol only if a small amount of mammalian tubulin was coassembled. We then carried out, on an analytical scale, affinity chromatography of yeast extracts comparing hybrid microtubules and microtubules made entirely of bovine tubulin. Seeing that these gave comparable results, we used the bovine tubulin to make preparative-scale columns with which we isolated a number of cell proteins, some of which we were able to show are associated with microtubules in the living yeast cell.

Isolation and Properties of Assembly-Competent Yeast Tubulin

Yeast Tubulin Purification. A flow chart for our yeast tubulin-purification procedure is shown in Figure 2, along with typical concentrations of protein from the disruption step to the final preparation. The protocol is given in detail in MATERIALS AND METHODS. Figure 3A shows the results of a stepwise elution from the DEAE column as described in the flow diagram; Figure 3B shows an immunoblot with anti-β-tubulin antibody of the gradient elution experiment from a DEAE column, in which the tubulin eluted from the DEAE column between 0.2 and 0.4 M NaCl. This result was used to determine the optimal stepwise salt elution conditions.

Inspection of Figure 3A suggests that the tubulin is ~50% pure after DEAE chromatography. Because yeast tubulin has been estimated to represent 0.05% of total soluble protein (Kilmartin, 1981), this represents a 1000-fold enrichment. After ammonium sulfate precipitation and desalting over the G-25 column, the concentration of tubulin was determined to be 4–4.5 mg/ml. At this stage, the desalted tubulin can be stored for ≥1 y at −70°C in monomer form without losing assembly competence.

Tubulin Assembly. As described in MATERIALS AND METHODS, yeast microtubules can be assembled after concentration with ammonium sulfate using freshly desalted tubulin or freshly thawed tubulin that had been

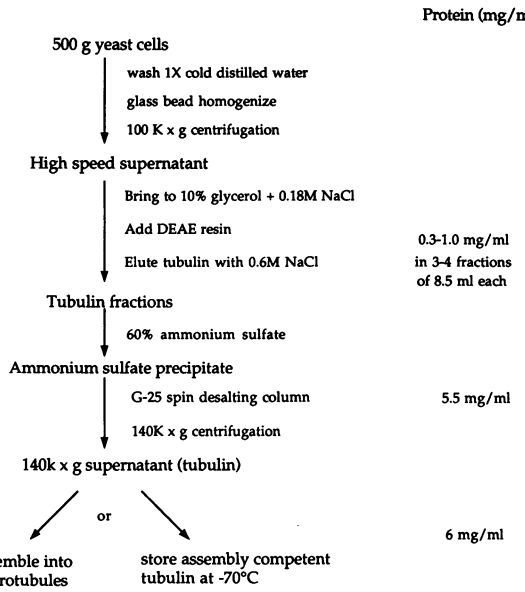

Figure 2. Flow chart for yeast tubulin purification.

desalted and stored at −70°C. GTP and DMSO were added to the tubulin monomer solution and the assembly reaction was incubated at 27°C for 20 min. To separate assembled microtubules from unassembled tubulin monomers, the assembly reaction was centrifuged at 140 000 × *g* at 27°C for 2 min. The pellet and supernatant fractions of the centrifugation were analyzed by one-dimensional SDS-page chromatography (Figure 4A). The lanes should accurately reflect the efficiency of the assembly reaction because the pellet was resuspended in a volume of assembly buffer equal to the supernatant volume and equal volumes of each loaded on the SDS-polyacrylamide gel. By inspection of this figure, we estimate the efficiency of this first round of assembly to be ~50%. The tubulin was then cycled through two more rounds of assembly each time with ~50% efficiency.

The microtubule assembly reaction is itself a significant tubulin purification step; as shown in Figure 4A, tubulin in the first pellet is ~90% pure. The protein band in Figure 4A that is labeled as tubulin was identified as such by immunoblotting experiments using several different antisera (see MATERIALS AND METHODS) and by direct protein sequencing, as described below. Protein determinations indicated that this procedure yields 7–10 mg of tubulin on average from 500 g of cells.

Sequence Analysis of Tubulin Fractions. To characterize further the proteins present in the assembly reaction pellet, two-dimensional gel isoelectric focusing and SDS page electrophoresis (Figure 4B) and protein sequence determination were carried out on the spots as described in MATERIALS AND METHODS. This analysis confirmed that the protein band at 56 kDa in Figure 4A contains the α- and β-tubulins and no other

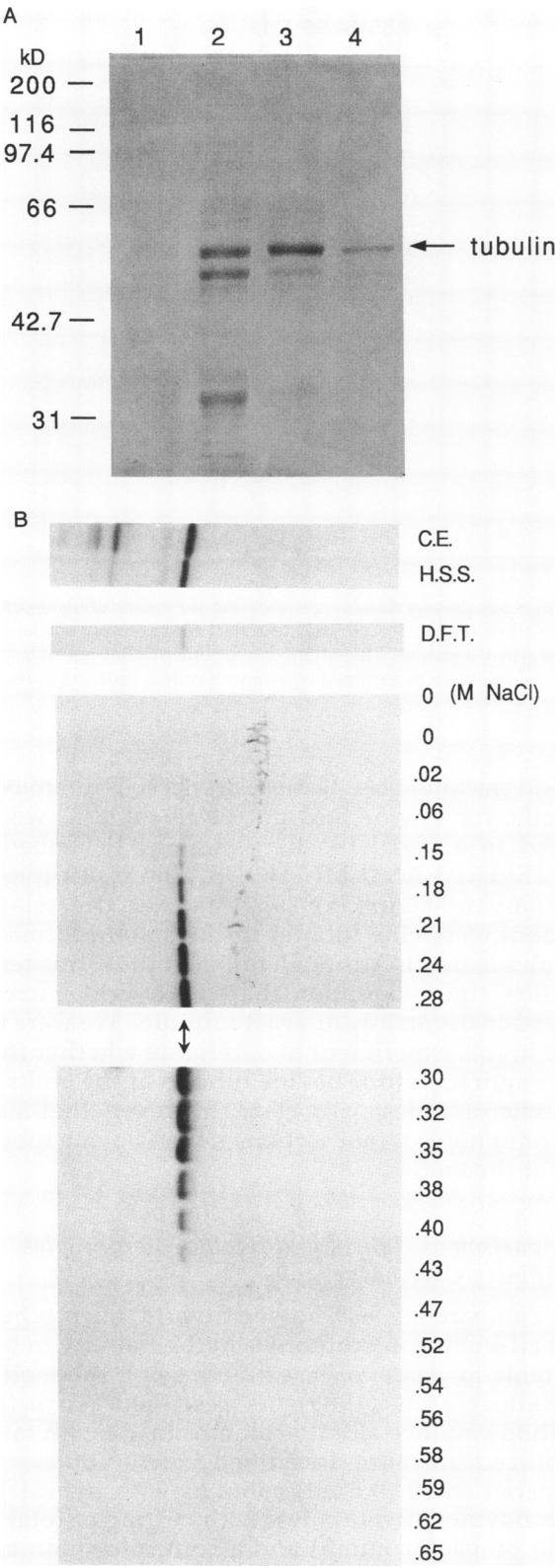

Figure 3. (A) A Coomassie blue-stained SDS 8.5% polyacrylamide gel showing four fractions from a 0.6 M NaCl step elution of protein from a DEAE column. The arrow identifies the protein band containing yeast α and β tubulin. (B) Immunoblot of the DEAE column fractions eluted with a 0–0.65 M NaCl gradient and probed with a yeast β tubulin-specific antibody. (The molar NaCl concentration is indicated for each fraction). Also shown for comparison with the DEAE fractions are aliquots of crude extract, high-speed supernatant, and DEAE column flow-through material. The arrows identify yeast β tubulin at 56 kDa.

detectable proteins. More importantly, the two-dimensional gel analysis and protein sequencing show that both α-tubulin isoforms, Tub1p and Tub3p, participate in the assembly reaction and both are present in the assembled microtubule polymer. Furthermore, the sequence results for the two α-tubulin protein spots at pI = 5.11 and pI = 4.96 (encoded by *TUB3* and *TUB1*, respectively) demonstrated that the transcripts encoding these proteins are spliced as predicted from the DNA sequences of the *TUB1* and *TUB3* genes (Schatz *et al.*, 1986a). The three clustered proteins at pI 4.65 were all determined by protein sequencing to be β-tubulins. The basis for the appearance of three *TUB2* β-tubulin isotypes is not yet known; however, the three β-spots represent changes in isoelectric point and not changes in molecular weight.

Finally, as a result of sequencing the β-tubulin protein spots, it was determined that the original report of the sequence of the *TUB2* gene is incorrect (Neff *et al.*, 1983). On inspection of the original DNA sequencing films, it was discovered that nucleotides #25, 35, and 477 were mistranscribed and should have been reported as #25, A; #35, G; and #477, T. As confirmed by the protein sequencing work presented in this paper, the correct amino acids at codon positions #9 and #12 are Thr and Cys, respectively.

Visualization of Assembled Yeast Microtubules. Two results confirm that the purified tubulin assembles into microtubules in vitro. First, the assembly products were collected on coverslips by centrifugation (Mitchison and Kirschner, 1984b) and were inspected by indirect immunofluorescence as described in MATERIALS AND METHODS. Figure 1A shows that filaments were formed in the assembly reaction. These microtubules were also examined by electron microscopy after negative staining. Twenty-five-nanometer filaments of apparently normal microtubule structure were observed (Figure 1B). In cross-section, normal protofilament patterns are observed in the polymer (Figure 1C). The tubulin polymers shown in Figure 1 formed during the assembly reaction because no such structures were observed if the assembly reaction was fixed before the 27°C incubation.

Stabilization of Polymerized Yeast Microtubules

The ability to stabilize microtubules against disassembly is useful and often essential for a number of different types of microtubule studies (Vallee and Collins, 1986). The conventional method for stabilizing in vitro-assembled microtubules purified from a number of sources such as bovine brain, procine brain, and *Drosophila* is to use the microtubule-stabilizing drug taxol. However,

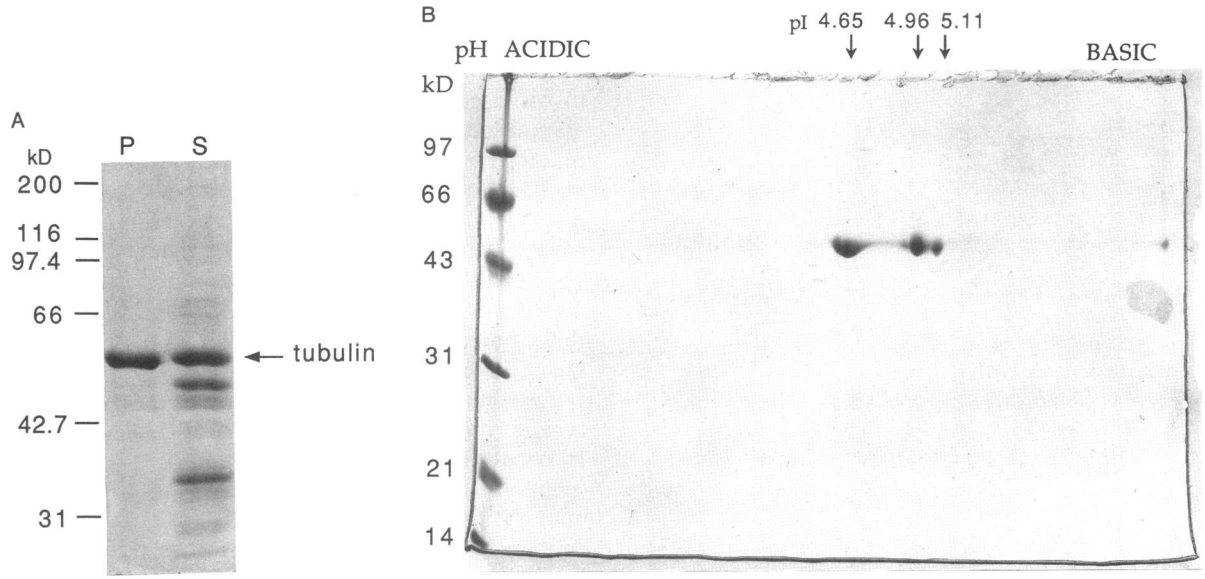

Figure 4. (A) Yeast microtubule assembly assay. Coomassie blue-stained SDS 8.5% polyacrylamide gel showing the microtubule assembly reaction pellet (P) and supernatant (S) proteins. Samples were prepared so as to allow the lanes accurately to reflect the assembly efficiency of the reaction. The protein band containing yeast α and β tubulin is indicated. (B) Coomassie blue-stained two-dimensional gel of a yeast microtubule assembly reaction pellet. The identities of β tubulin and α_1 and α_3 tubulins were determined by amino-terminal protein sequencing of each spot.

we were unable to stabilize yeast microtubules produced in vitro with taxol. Moreover, a taxol derivative, 10-deacetyl baccatine III, which also stabilizes in vitro-assembled microtubules isolated from most organisms (Lataste *et al.*, 1984), also did not stabilize our assembled yeast microtubules. In an attempt to assemble microtubules containing yeast tubulin that could be stabilized by taxol against disassembly, hybrid microtubules consisting of a mixture of tubulin from yeast and bovine brain in a ratio of six parts yeast tubulin to one part bovine brain tubulin were assembled. State of assembly was assayed by immunofluorescence with two antibodies: one specific for yeast β-tubulin (Bond *et al.*, 1986) and another, Amersham N.357, that binds to both yeast and bovine tubulin. To determine whether the hybrid microtubules could be stabilized, taxol was added to the assembly reactions and the reactions then diluted 1:10 in assembly buffer without taxol. After diluting the hybrid assembly reactions and incubating for 30 min to 3 d at room temperature, the microtubules remaining were sedimented onto coverslips and processed for immunofluorescence (Figure 5). Even after 3 d at room temperature, when pure yeast microtubules would long since have disassembled, hybrid microtubules are still seen. The background fluorescence on the coverslip increases after 3 d in each of the preparations for reasons not understood. The specificity of the antibodies allows the conclusion that the microtubules from the bovine/yeast mixture still present after 3 d contain yeast tubulin as well as bovine tubulin.

Figure 6 shows an experiment in which we attempted to localize the bovine and yeast tubulin in the stabilized hybrid microtubules. Because we lacked an antibody specific for bovine and not yeast tubulin, we resorted to a serial blocking strategy described in MATERIALS AND METHODS. The result (Figure 6; compare Figure 5) suggests that the relative amount of bovine tubulin in the stabilized microtubules is small. Although not definitive, this result fortifies the assumption that the hybrid microtubules consist mainly of yeast subunits. We were unable in this experiment to determine whether there is a requirement for bovine tubulin at the end(s) of the microtubules; it is clear, however, that some microtubules contain sections rich in bovine tubulin not at the end(s).

Comparison of Hybrid and Bovine Microtubules in Affinity Chromatography

We compared yeast/bovine hybrid microtubules and all-bovine microtubules in their ability to bind proteins in crude yeast extracts using the method of Kellogg *et al.* (1989). As described above, this method employs affinity columns made with taxol-stabilized in vitro-assembled microtubules. We prepared small (0.5-ml) columns with hybrid and with bovine microtubules (with ~1 mg of total tubulin on each column) and a control column containing BSA. These were used in an analytical scale experiment to compare the profiles of yeast proteins that bind to the microtubules. A high-speed (100 000 × *g*) supernatant of a crude yeast extract was passed over the columns in parallel. Total pro-

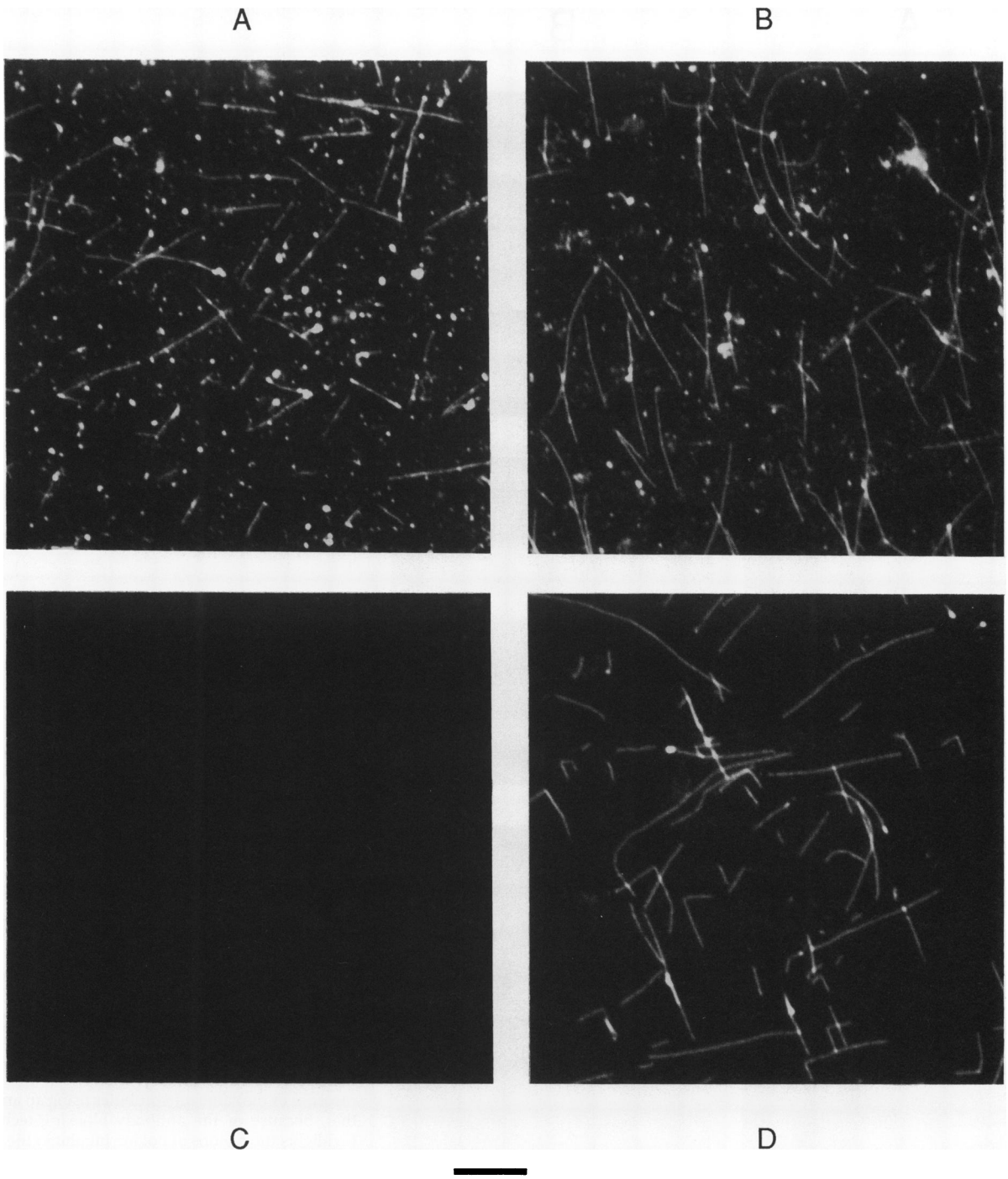

Figure 5. Immunofluorescence of taxol stabilized hybrid yeast:bovine (6:1) microtubules. (A and B) Hybrid microtubules pelleted onto coverslips after 3-d incubation of microtubules diluted 1:10 in PMI buffer without taxol. (C and D) Microtubules assembled with only bovine brain tubulin treated as described above. A and C were probed with a yeast-specific tubulin antibody. B and D were probed with YOL 1/34 that recognizes both yeast and bovine tubulins. Note in A the presence of gaps in the hybrid microtubules likely to contain only bovine tubulin. Bar = 6 μm.

tein bound to each column after loading and washing in the HEPES loading buffer (see MATERIALS AND METHODS) was extracted by boiling the resin in protein sample buffer and visualized by SDS-PAGE. The results (Figure 7) show that many proteins bind specifically to microtubules (yeast/bovine or bovine) as judged from the comparison with the BSA control column. However, there were little ob-

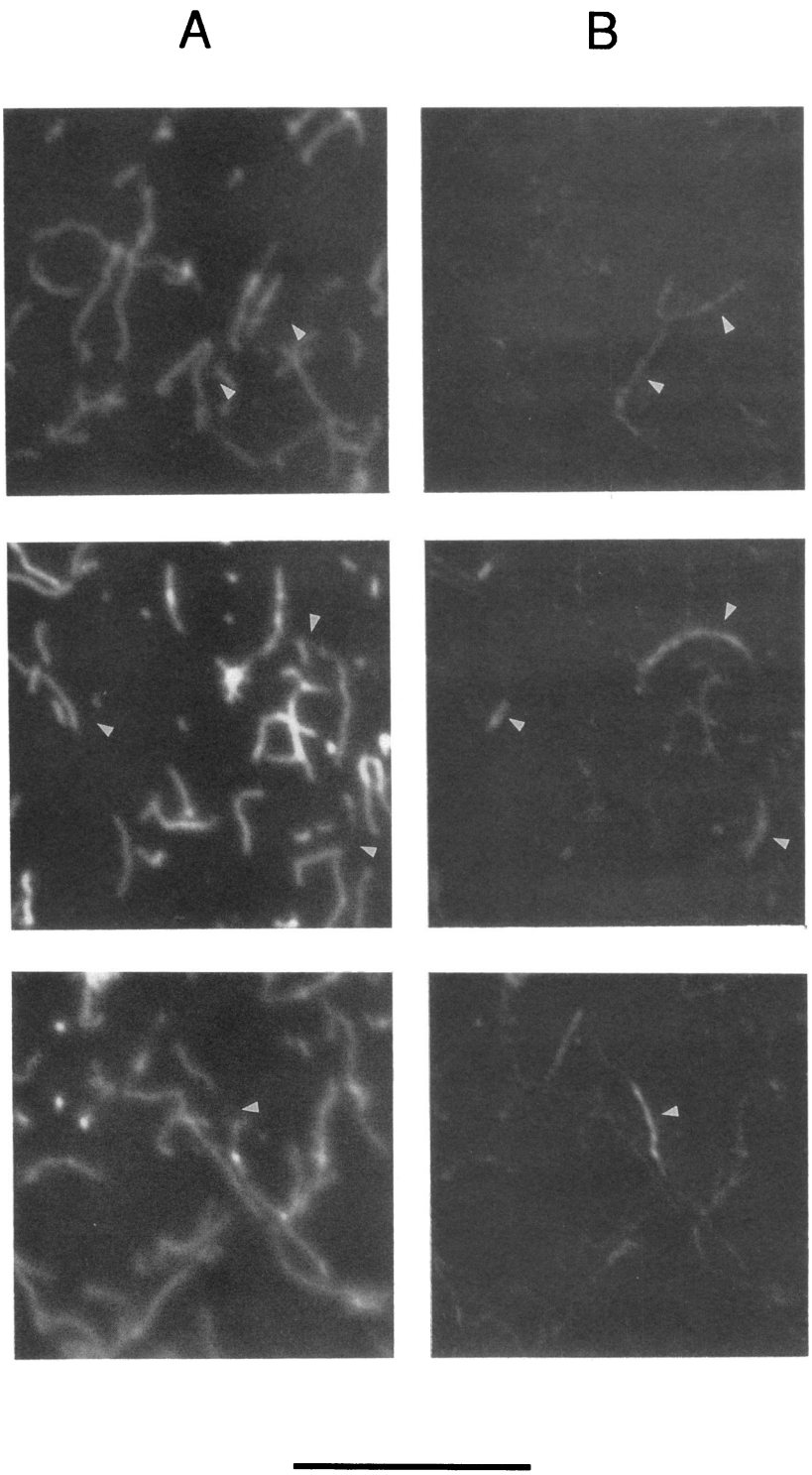

Figure 6. Double-label immunofluorescence localization of regions of bovine tubulin in yeast/bovine hybrid microtubules. Hybrid microtubules were incubated with yeast-tubulin specific rabbit antibodies and then incubated with 2° goat anti-rabbit rhodamine conjugated, 3° rabbit anti-goat, 4° goat anti-rabbit, 5° rabbit anti-goat, 6° Amersham mouse monoclonal anti-β tubulin, and finally 7° goat anti-mouse fluorochrome conjugated. The 2° goat anti-rabbit rhodamine conjugated sera was purified over mouse IgG on sephadex resin to eliminate cross-reactivity to mouse. The goat anti-mouse fluorochrome conjugated sera was purified over rabbit IgG on sephadex resin to eliminate cross reactivity to rabbit. A shows immunofluorescence staining with an antibody specific for yeast tubulin using a rhodamine-coupled secondary. B shows immunofluorescence of the same microtubules using an antibody that, because of the antibody blocking technique, highlights the regions of bovine tubulin in the hybrid microtubule. This antibody was visualized binding to a fluorescein-coupled secondary antibody. The bright regions in B indicated by arrowheads show discreet regions of bovine tubulin within the hybrid microtubules. Size bar at bottom is 10 μm.

vious differences in the complicated protein profiles between the hybrid and pure bovine columns. On this basis it seemed clear that one could isolate many (if not all) yeast microtubule-binding proteins using pure bovine tubulin.

Preparative Isolation of Yeast Microtubule-Binding Proteins

For preparative purposes, we used 16-ml columns containing about 30 mg phosphocellulose-purified bovine tubulin assembled in vitro and stabilized with taxol as

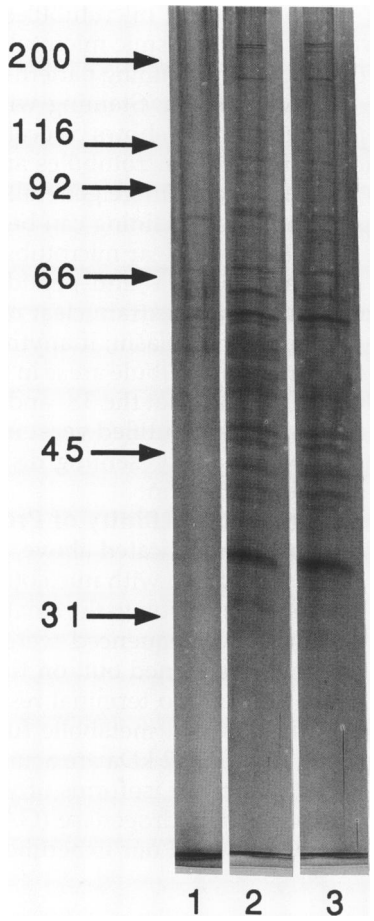

Figure 7. Analytical microtubule affinity chromatography experiment. A silver-stained SDS 8.5% polyacrylamide gel shows the total proteins eluted from the (lane 1) BSA control column, (lane 2) hybrid yeast/bovine microtubule column, and (lane 3) bovine microtubule column in the analytical scale experiment. Molecular size standards are expressed at the left in kDa.

described in MATERIALS AND METHODS. High-speed supernatants of yeast extracts were passed over a BSA-containing column and the eluate divided in half and passed in parallel over the microtubule column and a second BSA column. The proteins eluted from these latter columns by 5 mM ATP, 2 mM GTP (hereafter "nucleotide elution"), 0.1 M KCl (hereafter "low salt"), and 0.5 M KCl (hereafter "high salt") were analyzed by SDS-PAGE followed by silver staining. The results are shown in Figure 8. A distinct set of proteins is retained on the microtubule column but not on the BSA control column run in parallel. Detection of the proteins that eluted with buffer containing low or high salt on SDS-PAGE gels required silver staining.

Small GTP Binding Proteins. Because genetic studies indicated that the putative GTP binding protein encoded by the *CIN4* gene is involved in microtubule function in yeast (Hoyt *et al.*, 1990; Stearns *et al.*, 1990a), we were motivated to determine whether Cin4p or any other small GTP binding proteins might be among the proteins that bind specifically to our microtubule columns. Filters containing immobilized protein fractions resolved by SDS-PAGE were probed with α-^{32}P GTP (Lapetina and Reep, 1987). Proteins eluted by all three elution conditions were examined. GTP binding proteins were only retained by the microtubule column and not the BSA column. However, only the fractions eluted with nucleotide were found to contain GTP binding proteins (Figure 9).

The microtubule column profile of GTP binding proteins is distinct from the profile of GTP binding proteins in a total extract or in any of the flow-through fractions in Figure 9. The proteins bound to the microtubule column include two at 27 and 25 kDa that are greatly enriched on the microtubule column compared with the other extracts examined. Further, there are GTP binding proteins present in the protein extracts that are completely absent from the microtubule elution fraction (proteins at about 18 and 38 kDa); these must have quantitatively flowed through the microtubule column. Finally, there is a group of proteins at ~30 kDa that bind to some extent to the microtubule column but are not enriched over the extract fractions. We conclude that the 27- and 25-kDa proteins bind to the microtubule column with high affinity.

Preliminary immunoblot analysis with specific antibodies made it unlikely that the 25- and 27-kDa GTP binding proteins might be the *YPT1*, *RAS*, or *ARF* proteins. Apparently these flow through the microtubule column. To identify the GTP-binding proteins we carried out amino-terminal protein sequencing as described in MATERIALS AND METHODS. It was necessary to concentrate these proteins (described in MATERIALS AND METHODS) for sequencing because the protein bands corresponding to the GTP binding activities were not visible even by silver staining. These sequences were compared with protein sequences in existing databases. No match was found for the 27-kDa band. However, 21 amino acids of the 25-kDa protein were found to match exactly the amino terminal sequence of the yeast *SAR1* protein (Nakano and Muramatsu, 1989). The yeast *SAR1* gene is a member of the small GTP-binding protein superfamily and was originally identified as a multicopy and low-copy plasmid suppressor of the secretory mutant *sec12-1*, *sec12-3*, and *sec12-4*. Our protein sequence data also proves that the *SAR1* mRNA is indeed spliced as proposed by Nakano and Muramatsu (1989).

Identification of other Proteins Eluted with Nucleotide. The amino termini of seven additional proteins eluted from the microtubule column with nucleotide were determined. The pattern of elution is shown in Figure 10, which shows a Coomassie blue-stained SDS-PAGE analysis of the eluate. About 22 amino terminal amino acids were determined for each protein. Two of the sequences, from proteins with an apparent molec-

ular size of 38 and 50 kDa, are not represented in the protein databases. As these were judged to be excellent candidates for new microtubule-associated proteins, anti-peptide antibodies were raised to facilitate immunofluorescence experiments. Crude antisera raised in rabbits were affinity-purified on peptide affinity columns as described in MATERIALS AND METHODS. The affinity-purified antibodies were tested for their ability to recognize the intact protein by immunoblot analysis. Figure 11 shows this result; the nucleotide elution fractions from the microtubule column were analyzed by SDS-PAGE, transferred to nitrocellulose, and probed with the affinity-purified antibodies. It can be seen that both the anti-50 and anti-38 kDa affinity-purified anti-peptide antibodies recognize the intact 50- and 38-kDa proteins, respectively. When yeast crude extracts were analyzed in this manner, the anti-50-kDa peptide antibody recognized only a single protein at 50 kDa. However, the anti-38-kDa peptide antibody recognized the 38-kDa protein and three additional proteins at molecular weights 28, 60, and 170 kDa.

Localization of the 38 and 50 kDa Proteins in the Living Cell. Wild-type yeast cells were processed for immunofluorescence and probed with the affinity-purified anti-peptide antibodies and also with anti-tubulin antibodies. The affinity-purified antisera showed that both proteins purified on the microtubule column in vitro associate with the microtubule cytoskeleton in vivo (Figures 12 and 13). The 38- and 50-kDa proteins colocalize with the intranuclear microtubules as well as with the extranuclear cytoplasmic microtubules.

However, the fluorescent staining patterns are slightly different for the two antisera. Staining with the anti-38-kDa anti-peptide antibody shows colocalization with the intra- and extranuclear microtubules and stain appears also to be concentrated more generally in the nucleus, although the nuclear staining can be clearly distinguished from the intranuclear microtubule staining. In contrast, the anti-50-kDa anti-peptide antibody brightly stains the intra- and extranuclear microtubules and diffusely stains the cytoplasm; if anything, stain is excluded from the nonmicrotubule areas in the nucleus. These experiments indicate that the 38- and the 50-kDa proteins are heretofore unidentified yeast microtubule-binding proteins possibly representing distinct classes of microtubule-binding protein.

Evidence for Microtubule-Affinity of Proteins With Metabolic Functions. As indicated above, only two of the seven proteins that eluted with nucleotide from the microtubule column (in addition to the small GTP binding proteins) and that we sequenced were previously unknown. The other five turned out, on the basis of a perfect match in all 22 amino terminal residues, to be yeast proteins with a known metabolic function. The proteins migrating at about 32 kDa are actually a triplet of proteins that are the three isoforms of yeast glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The isolation of yeast GAPDH in our experiments was ex-

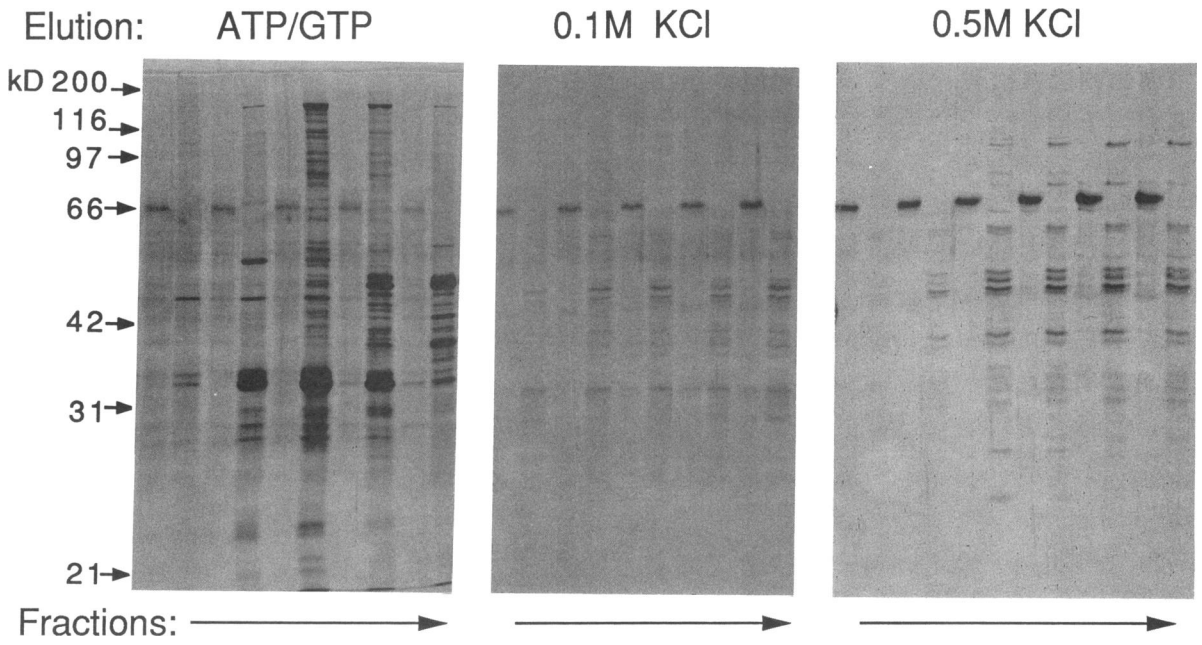

Figure 8. Left, Preparative scale purification of *S. cerevisiae* MAPs eluted with nucleotide (ATP/GTP); middle, low salt (0.1 M KCl); or right high salt (0.5 M KCl). Shown are silver-stained fractions of an SDS 8.5% polyacrylamide gel. In all three panels, the fractions are loaded in pairs: (left lane of pair) BSA control column elution and (right lane of pair) bovine microtubule column elution. Molecular size standards are expressed at the left in kDa.

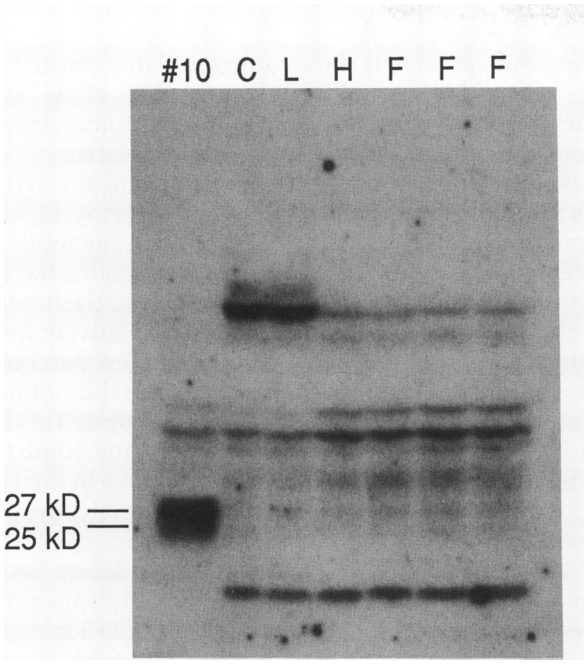

Figure 9. GTP binding activity assay. Proteins were separated on an SDS 8.5% polyacrylamide gel, transferred to nitrocellulose, and then incubated in the presence of α^{32}P-GTP. Shown are the GTP binding activities from nucleotide elution fraction from the microtubule column (lane #10), yeast crude extract (lane C), low speed supernatant (lane L), high-speed supernatant (lane H), large BSA column flow-through, small BSA column flow-through, and microtubule column flow-through (lane F, F, F, left to right, respectively). Size of the two GTP binding proteins that bound to the microtubule column are indicated.

pected and serves as an internal control for the effectiveness of the microtubule affinity columns because GAPDH has independently been identified by two groups as a 35-kDa MAP in brain tubulin cycling experiments. Before its identification as GAPDH, it was shown that this MAP can bind to and bundle microtubules in the absence of ATP (Kumagai and Sakai, 1983; Huitorel and Pantaloni, 1985); in the presence of ATP, it dissociates from microtubules.

In our experiments, the high-speed supernatant was depleted of ATP by the addition of apyrase; it is this condition that apparently favored binding of GAPDH. Yeast has three isoforms of GAPDH encoded by three different genes, *TDH1*, *TDH2*, and *TDH3* (McAlister and Holland, 1985a,b). *TDH2* and *TDH3* encode the majority of the activity (~85–90%). The minor isoform encoded by the *TDH1* gene cannot functionally substitute for absence of the major isoforms. Although all three isoforms bind to the microtubule column, the minor isoform is enriched over the major isoforms. This can be seen most clearly in the elution fractions shown in Figure 10 where the lower band at 35 kDa is the minor GAPDH isoform encoded by *TDH1* and the upper band is actually a doublet of the major isoforms encoded by *TDH2* and *TDH3*. See Figure 10 (right) for a comparison of the relative abundance of the isoforms in a crude yeast extract and in the peak elution fraction 7.

The 160-kDa protein in fractions 7 and 8 (Figure 10) is the product of the yeast *ARO1* gene, a pentafunctional enzyme involved in aromatic amino acid biosynthesis (Duncan *et al.*, 1987). It is apparent by inspection of the data presented in Figure 10 that Aro1p is a nonabundant protein that is highly enriched on the microtubule column. Finally, the protein with apparent size of 49 kDa in fractions 8 and 9 is yeast mitochondrial citrate synthase encoded by the *CIT1* gene (Rosenkrantz *et al.*, 1986).

DISCUSSION

The first series of experiments presented above show that pure tubulin from *S. cerevisiae* can be assembled in vitro and that the assembled tubulin contains the two α and one β subtypes specified by the three tubulin genes of this organism (Neff *et al.*, 1983; Schatz *et al.*, 1986a,b). Our characterization of these microtubules showed them to be standard in morphology (Amos and Baker, 1979); the only remarkable findings were some indication of post-translational modification of the β

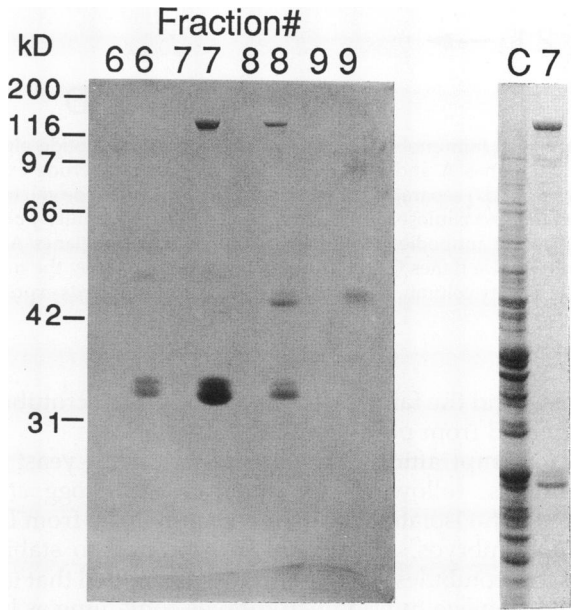

Figure 10. Coomassie blue-stained SDS 8.5% polyacrylamide gel showing the proteins eluted with nucleotide from the microtubule affinity column (right member of pair) and the BSA control column (left member of pair) loaded in pairs. Fraction numbers across the top of the panel correspond to the peak protein eluted fractions and are also the same fractions shown by silver stain in Figure 8 that also shows fraction #10 of the nucleotide elution. On the right side, two lanes show a comparison of Coomassie blue-stained protein profile of yeast crude extract (lane C) and the profile of nucleotide elution fraction 7 from the microtubule column (lane labeled 7). Molecular size markers are expressed in kDa.

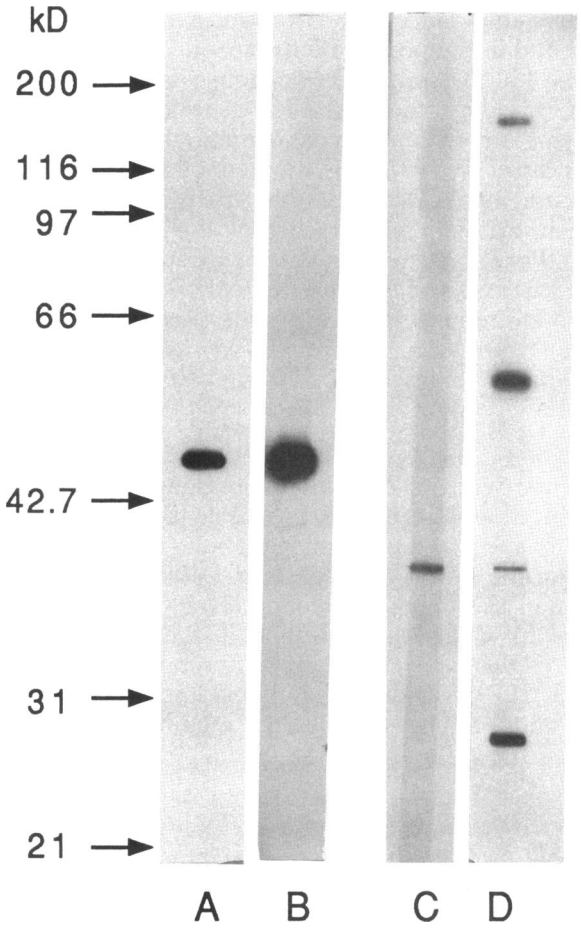

Figure 11. Immunoblot analysis of proteins in nucleotide elution fractions (lanes A and C) and proteins in yeast total crude extract (lanes B and D) separated on an SDS 8.5% polyacrylamide gel, transferred to nitrocellulose, and then incubated with the affinity-purified anti-peptide antibodies recognizing either the 50 kDa (lanes A and B) and 38 kDa (lanes C and D) yeast MAPs identified by the microtubule affinity column. Shown at the left are size standards expressed in kDa.

subunit and the failure of taxol to stabilize microtubules assembled from pure yeast tubulins.

To attempt affinity chromatography with yeast microtubules, following the example of Kellogg *et al.* (1989), who isolated many interesting MAPs from *Drosophila* embryos, a way had to be found to stabilize yeast microtubules. To this end, we discovered that taxol would stabilize hybrid microtubules containing as little as 1:6 admixture of bovine brain tubulin:to yeast tubulin. Characterization of the hybrids showed that the assembled microtubules contain mainly yeast subunits but are stabilized indefinitely by taxol, as are microtubules of bovine origin. However, yeast tubulin comprises ~0.05% of yeast protein whereas bovine brain tubulin can be as much as 20% of cell protein. This consideration motivated us to compare the pattern of yeast proteins bound by the hybrid and pure bovine microtubules. The result, as shown above, suggested that most, if not all, yeast MAPs would bind as well to either kind. Therefore, we continued our affinity chromatography work with the more available bovine material.

Before leaving the subject of yeast:bovine (6:1) hybrid microtubules, three observations are worthy of note. First, the ability of taxol to stabilize these microtubules is remarkable in view of the fact that so small a fraction of the tubulin was of bovine origin. Second, the tubulins of the two species seem to polymerize relatively freely with each other; we observed relatively few patches of bovine tubulin and the stabilized tubules did not have visible stretches of bovine tubulin concentrated at their ends. Third, despite these observations, the admixture of bovine subunits caused a marked change in the temperature-dependence of microtubule assembly: the pure yeast and bovine subunits assemble best at 27° and 37°C, respectively, whereas the 1:6 mixture works equally well at either temperature. It would appear that the bovine subunits are able to contribute kinetically more than one might expect from the stoichiometry. This idea is supported by our observation that bovine tubulin is able to drive yeast tubulin preparations incapable of self assembly (possible due to the presence of a yeast tubulin specific inhibitor) into polymerized hybrid microtubules.

We reasoned that yeast MAPs, like the *Drosophila* embryo MAPs (Kellogg *et al.*, 1989), would not be abundant proteins and decided to use the microtubule affinity chromatography technique to identify and isolate yeast MAPs. Because a similar complex elution profile of yeast proteins was observed, as discussed above, with both taxol-stabilized bovine and yeast:bovine (6:1) microtubules, we made preparative columns with bovine material. About 25 proteins bound specifically to the microtubule affinity column and eluted under different buffer conditions. Three of the nine proteins whose amino-terminal sequence we determined turned out to be unknown in the standard sequence databases. The discovery of novel proteins in this experiment was expected because the sequences of very few MAPs are known: tau, MAP2, MAP1B, kinesin, dynein, and dynamin (Lee *et al.*, 1988; Lewis *et al.*, 1988; Noble *et al.*, 1989; Yang *et al.*, 1989; Obar *et al.*, 1990; Gibbons *et al.*, 1991; Ogawa, 1991). Given the number of cellular functions that the microtubule cytoskeleton carries out and given the requirement for regulation of the timing and positioning of these processes, the involvement of 25 or more proteins in addition to tubulin was to be expected.

By raising anti-peptide antibodies directed against the amino-terminal sequences of two of the novel proteins (50 and 38 kDa), we recovered and were able to affinity-purify antibodies that specifically bind to the proteins. In the case of the 50-kDa species, only a single species was seen on immunoblots in both purified fractions and crude extract whereas the anti-38-kDa antibody rec-

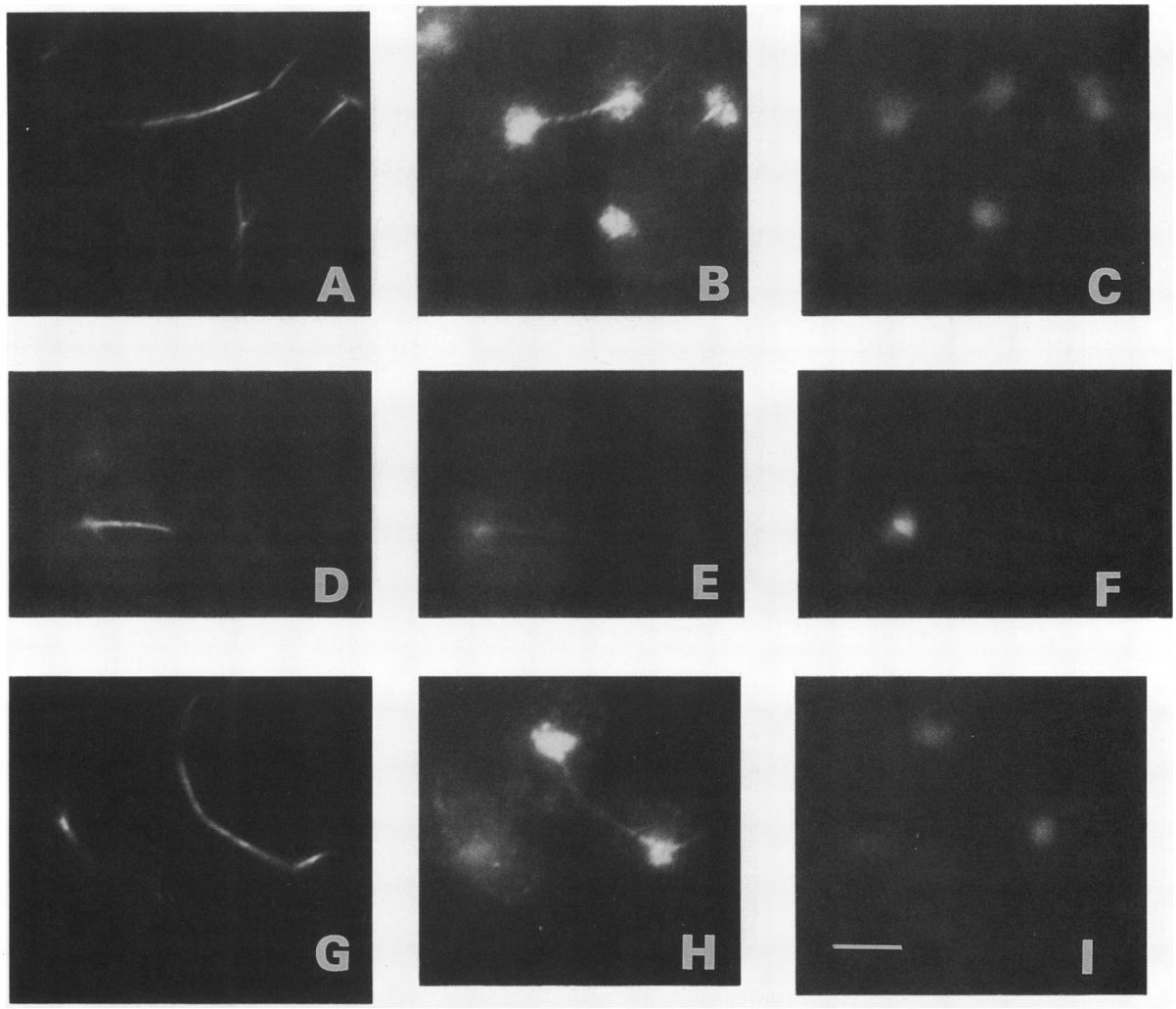

Figure 12. Double-label immunofluorescence of microtubules and the 38-kDa MAP. (A, D, and G) Rhodamine anti-tubulin immunofluorescence. (B, E, and H) Fluorescein anti-38-kDa MAP immunofluorescence of the same cells shown in A, D, and G, respectively. (C, F, and I) DAPI staining of the same cells as shown in A, D, and G, respectively. Shown in panels D, E, and F is an unbudded cell with a single projection of cytoplasmic microtubules. Bar in I is 5 μm.

ognizes one species in the microtubule column fractions and three additional species in the crude extract. Using these antibodies, we could show for both proteins association with microtubules in the living cell by immunofluorescence microscopy.

In the case of the 50-kDa protein, the association is unambiguous; one sees strong staining coincident with the tubulin cytoskeleton. The only other staining one sees is weak and diffused throughout the cytoplasm. This pattern could simply reflect binding to unassembled or nascent proteins. More information on the possible roles of this now well-defined MAP await further analysis including cloning, sequence, and genetics that is underway.

The case of the 38-kDa protein is more complex because of the additional species detected by the antibody in immunoblots of the crude extract. Nevertheless, at least one of the species, presumably the single one (38 kDa) that bound to the microtubule columns in vitro, colocalizes with tubulin. At least one of these proteins (although not necessarily the 38-kDa species) seems to be concentrated in the nucleus. It should be noted that we have not ruled out the possibility that all the species detected by the anti-38-kDa antibody are related to each other. The issues concerning both function(s) and relationships among the proteins recognized by the anti-38-kDa antibody should be resolved by further analysis that is also underway.

Our ability to find, in such a straightforward way, novel MAPs that associate with the cytoskeleton in vivo as well as in the test tube is a strong endorsement of the approach of Kellogg *et al.* (1989). Our experience verifies the simplicity and value of immobilized fully polymerized microtubules in place of the classical as-

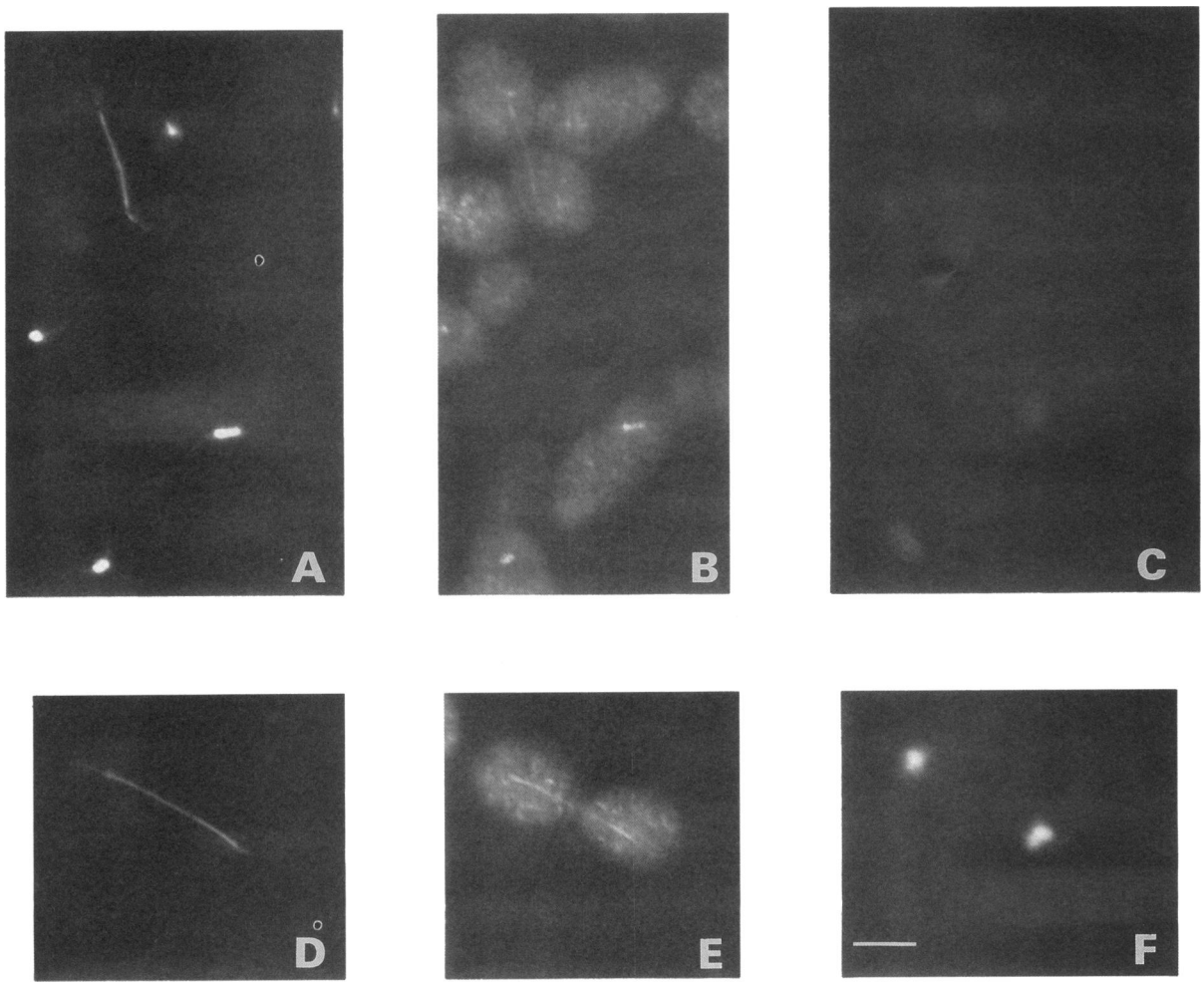

Figure 13. Double-label immunofluorescence of microtubules and the 50 kDa MAP. (A and D) Rhodamine anti-tubulin immunofluorescence. (B and E) Fluorescein anti-50-kDa MAP immunofluorescence of the same cells shown in A and D, respectively. (C and F) DAPI staining of the same cells shown in A and D, respectively. Bar in F is 5 μm.

sembly-disassembly cycling experiments. It is notable that we used bovine microtubules to isolate yeast proteins, having shown that mainly yeast microtubules give a similar pattern. It will soon become clear, when cloning and sequencing efforts proceed further, whether the same new MAPs are being found in *Drosophila* and yeast.

In addition to the two proteins above, two small GTP binding proteins were found to bind to the microtubule affinity column and to elute with nucleotide. The question of whether small GTP binding proteins were present in the elution fractions from the microtubule column arose because genetic experiments indicated that the putative GTP binding protein encoded by *CIN4* is involved in microtubule function (Hoyt *et al.*, 1990; Stearns *et al.*, 1990a). We found that four small GTP binding proteins bind to microtubule columns and that two of these four (25 and 27 kDa in size) bind very specifically and are highly enriched. The amino terminal sequence of the 27-kDa protein was not present in the sequence databases, whereas an exact match was found for the 25-kDa protein with the amino terminus of the *S. cerevisiae* protein encoded by the *SAR1* gene.

The *SAR1* gene was originally identified as a multicopy suppressor of *sec12* and also *sec16* mutations (Nakano and Muramatsu, 1989), an observation suggesting a role in the yeast secretory pathway. Nakano and Muramatsu (1989) showed that this is true by depleting yeast cells of Sar1p and then demonstrating that a secretion defect results. Our discovery that Sar1p is retained and greatly enriched on a microtubule column in the presence of detergent suggests that yeast microtubules might also play a role in the secretory process. In other eukaryotes, the microtubule cytoskeleton is involved in various aspects of protein secretion including directed vesicle transport, establishment of cellular organelle architecture such as organization of the endoplasmic reticulum and Golgi network, (reviewed in

Kelly, 1990), and in the recycling pathway from the Golgi to the endoplasmic reticulum (Lippincott-Schwartz *et al.*, 1990). In yeast, it has been demonstrated that microtubules are not essential for protein secretion (Huffaker *et al.*, 1988; Makarow, 1988). It remains to be determined whether microtubules might function in the secretory process in yeast to ensure, for example, the efficiency of the process.

The third class of proteins isolated on the microtubule affinity column are metabolic enzymes. Previously, a "35-kDa" MAP from mammalian brain tubulin cycling experiments was isolated by two groups (Kumagai and Sakai, 1983; Huitorel and Pantaloni, 1985) and was shown to be GAPDH. This 35-kDa MAP is able to bind to and bundle microtubules in the absence of ATP but dissociates from the microtubules in the presence of ATP. We found that yeast GAPDH was bound to our microtubule columns. In our experiments, the ATP effect seen with other species may also have been repeated, as our yeast protein extract was depleted of ATP with apyrase before passage over the microtubule affinity column.

The yeast GAPDH proteins are encoded by three genes: *TDH1*, *2*, and *3*. All three isoforms are eluted from the microtubule columns with ATP. In yeast, the three *TDH* genes are highly homologous, but there appear nevertheless to be functional differences between them. GAPDH comprises as much as 10% of yeast soluble protein; *TDH2* and *TDH3* genes encode the majority of the GAPDH activity (~85%). The minor isoform encoded by the *TDH1* gene is not able to substitute for the absence of both of the major isoforms. Although the major isoforms Tdh2p and Tdh3p bind to the column, it is the minor (ca. 15%) isoform Tdh1p that is enriched most highly on the microtubule column (Figure 10).

The other two enzymes found on the column are the pentafunctional enzyme encoded by the *ARO1* gene and the citrate synthase isoform encoded by the *CIT1* gene (Rosenkrantz *et al.*, 1986; Duncan *et al.*, 1987). Although the two isoforms of yeast citrate synthase are quite homologous (Rosenkrantz *et al.*, 1986), only the *CIT1*-encoded protein bound to the column. This isoform is encoded by a nuclear gene but is localized to the mitochondria and must presumably pass through the cytoplasm to get to its destination. Moreover, recent results of Azpiroz and Butow (personal communication) suggest that yeast citrate synthase is transported vectorially across yeast zygotes in a manner distinct from mitochondrial DNA, a result that might be consistent with our finding of an association of the enzyme with microtubules.

In addition to identifying MAP35 as GAPDH, other cytoskeleton-associated proteins have been identified previously that have roles apparently distinct from the functions of the cytoskeleton. For example, a mitotic apparatus-associated 51-kDa protein has extensive homology to elongation factor 1α and will react with antibodies directed against EF-1α (Ohta *et al.*, 1990); furthermore, the c-*mos* proto-oncogene product has been shown to colocalize with tubulin in the spindle of transformed NIH 3T3 cells (Zhou *et al.*, 1991). However, questions still remain about the functional nature of the association with the microtubule cytoskeleton with other types of cellular proteins.

There are diverse possible explanations for the binding of metabolic enzymes to cytoskeletal elements. One possibility, of course, is that these interactions are completely adventitious and bind to tubulin because of some physical property like charge or hyrophobicity. However, there may well be more interesting reasons, and the finding that GAPDH bundles as well as binds offers some hope of a more interesting reason. The preferential enrichment of the minor isoform is also suggestive. One of the best examples of a known metabolic enzyme performing a function other than, or in addition to, its known metabolic role is the lens crystallin protein, which recently was found to be lactate dehydrogenase (Graeme and Piatigorsky, 1987; Hendriks *et al.*, 1988).

We do not propose that all associations of a metabolic enzyme with cytoskeletal elements are due to a structural role for the enzyme(s). It may equally well be that association with a cytoskeletal element serves to aid in the subcellular distribution of the enzyme. This type of mechanism would explain why the mitochondrial form of the yeast citrate synthase, Cit1p, bound to the microtubule column but the cytoplasmic form of the protein did not, even though the two proteins are 75% identical (Rosenkrantz *et al.*, 1986). It is important to remember that only a limited amount of information is obtained about the function of a protein when only a limited number of questions is asked about that protein's function.

In conclusion, the affinity chromatography technique used in this study has two advantages over other approaches. First, it is possible to identify low-abundance proteins that interact with microtubules. Second, the technique makes it possible to identify proteins whose in vivo function is duplicated by the functions of other proteins in the cell. The duplication of function by two proteins is not uncommon in yeast (examples include Kataoka *et al.*, 1984; Schatz *et al.*, 1986a,b; Basson *et al.*, 1987; Stearns *et al.*, 1990b). A protein whose function is duplicated by other proteins can be extremely difficult to identify initially by genetic approaches, but once found it can be easily studied genetically.

We have found that bovine microtubules stabilized with taxol allow the isolation of a large number and variety of yeast proteins that associate with microtubules. We have confidence that this method enriches for bona fide MAPs because the first two new proteins we studied colocalize with the tubulin cytoskeleton in the living cell. In addition, we found a number of small GTP binding proteins, one of which suggests a con-

nection between microtubules and protein secretion in yeast. Finally, we found a number of metabolic enzymes. In each of these cases, the finding of the association will allow genetic and physiological and ultimately biochemical studies of microtubule function in vivo as well as in vitro.

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